

## Immunogenicity and cross reactivity indices of *Streptococcus equi* subsp. *equi* strains isolated from cases of Strangles and commercial vaccines

Imunogenicidade e índices de reatividade cruzada de cepas de *Streptococcus equi* subsp. *equi* isoladas de casos de Adenite Equina e vacinas comerciais

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### ABSTRACT

Horse Strangles appears frequently in animals vaccinated with commercial bacterins in Rio Grande do Sul, Brazil. Aiming to know the antigenic relationships of strains recovered from sick animals among them and with two vaccines profusely used in the state, bilateral cross reactivity indices (CRI) were estimated. In addition, the immunogenicity of vaccines prepared with field isolates and commercial vaccines was tested in mice. Antibody titers were measured by ELISA and expressed as seroconversions. Thirteen strains of *Streptococcus equi* subsp. *equi*, nine classified biochemically as typical and other four as atypical strains, were recovered from 35 sick horses belonging to 10 herds of Rio Grande do Sul, Brazil. The strains recovered from sick horses showed very close CRI, suggesting antigenic homogeneity among them, but not with the vaccinal strains. A vaccine produced with an atypical strain induced the highest seroconversion, 9.4, while two produced with typical strains were poorly-immunogenic. The commercial vaccines were less immunogenic than five and four vaccines produced with field strains, inducing seroconversions of 2.6 and 3.8, respectively.

**Key words:** horse Strangles, *Streptococcus equi* subsp. *equi*, cross reactivity indices, vaccines.

### RESUMO

A Adenite Equina é freqüente em animais vacinados com bacterinas comerciais no Rio Grande do Sul. Com o objetivo de determinar as relações antigênicas entre cepas isoladas de casos clínicos e duas vacinas amplamente utilizadas no Estado, foram determinados os índices de reatividade cruzada bilateral (IRC) entre elas. Também foi determinada a imunogenicidade em camundongos de vacinas

preparadas com cepas de campo e de vacinas comerciais. Os títulos de anticorpos foram determinados por ELISA e expressos como soroconversão. Foram isoladas 13 cepas de *Streptococcus equi* subsp. *equi* de 35 casos clínicos de Adenite Equina em 10 diferentes rebanhos do Rio Grande do Sul, Brasil, das quais nove foram classificadas bioquimicamente como típicas e outras quatro como atípicas. As cepas de campo apresentaram elevada reatividade cruzada, sugerindo homogeneidade antigênica entre elas, mas não com as cepas vacinais. Uma vacina produzida com cepa atípica induziu soroconversão de 9.4, enquanto duas vacinas produzidas com cepas típicas foram pouco imunogênicas. As vacinas comerciais induziram soroconversões de 2.6 e 3.8, menores que quatro e cinco vacinas produzidas com cepas de campo, respectivamente.

**Palavra-chave:** Adenite Equina, *Streptococcus equi* subsp. *equi*, Índice de Reatividade Cruzada, Vacinas.

### INTRODUCTION

Horse Strangles is a highly transmissible infectious disease of horses caused by *Streptococcus equi* subsp. *equi* (*S. equi* subsp. *equi*), a Gram-positive streptococcus belonging to Lancefield group C (FARROW & COLLINS, 1984). It affects horses of all ages, although is more frequent among young animals (TIMONEY et al., 1997), producing mucopurulent nasal discharges, lymphadenitis and guttural pouch empyema (SWEENEY, 1993). The disease has very high morbidity and low mortality, and its economic impact is

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due to veterinarian expenses and to the necessity to restrain the animals from their activities during several weeks. Outbreaks may last months or even years, mainly in large horse populations where animals are frequently introduced (CHANTER, 1997).

The clinical diagnosis of the disease is easy, but the recovery of *S. equi* subsp. *equi* is hampered by opportunistic bacteria such as *S. equi* subsp. *zooepidemicus* and *S. dysgalactiae* subsp. *equisimilis* (KUWAMOTO et al., 2001), that are frequently isolated from typical cases of the disease. Typical strains of *S. equi* subsp. *equi* do not ferment lactose, sorbitol and trehalose, while lactose and sorbitol are fermented by *S. equi* subsp. *zooepidemicus* and lactose and trehalose by *S. dysgalactiae* subsp. *equisimilis*, (EUZÉBY, 2005). In addition, *S. equi* subsp. *equi* carbohydrate-fermenting strains, known as atypical strains, add confusion to the bacteriologic diagnosis (GRANT et al., 1993), demanding supplementary tests for their accurate identification.

*S. equi* subsp. *equi* produces two antigenically different proteins M (HARRINGTON et al., 2002), as well as other structural or secreted antigens that may participate in its pathogenicity and immunogenicity (FLOCK et al., 2004). The inappropriate selection or manipulation of vaccinal strains may be involved in the low protection conferred by some commercial vaccines, making the study of the antigenic relationships among strains useful when candidate strains for vaccines are being chosen.

The strains isolated from field cases of Strangles and two commercial vaccines were studied through Western Blot and ELISA using recombinant *S. equi* protein M as antigen, and the bilateral cross reactivity index (CRI) among them was estimated. CRI, that estimates the level of cross protection induced by strains of the same antigen (PEREIRA, 1977), was profusely used to subtype Foot and Mouth Disease Virus. It was considered that a CRI of 70 is the threshold to classify strains as identical (BROOKSBY, 1968). CRIs were also used to estimate antigenic relationships of different antigenic groups of *Bordetella bronchiseptica* (OLIVEIRA & GIL-TURNES, 1988) and *Moraxella bovis* (CONCEIÇÃO et al., 2003), the etiological agents of Atrophic Rhinitis of swine and Bovine Infectious Keratoconjunctivitis, respectively.

The objective of this research was to study the immunogenicity and the antigenic relationships among strains recovered from clinical cases of Strangles and two strains used in commercial vaccines widely used in Brazil.

## MATERIAL AND METHODS

### Samples

Nasal discharges were collected from 35 horses with initial signs of Strangles, that belonged to 10 herds of three municipalities of Rio Grande do Sul, Brazil. The samples were sown on 10% sheep blood agar and incubated for 48 h at 37°C. Colonies of Gram positive, catalase negative cocci, were suspended in sterile saline and grown in Phenol Red Broth base (Difco, Detroit, MI, USA) containing 1% trehalose, sorbitol or lactose, with or without 10% sterile horse serum devoid of antibodies against streptococci. The isolates were also tested by the API 20 STREP (BioMérieux Brasil S.A., São Paulo, SP), following manufacturer's instructions. Fermentation of carbohydrates was read up to 48h of culture at 37°C, and the API STREP reactions at 4 and 24h of culture. The strains were classified following EUZÉBY (2005).

### Vaccines

Ten out of 13 strains of *S. equi* subsp. *equi* isolated from field cases, were used to prepare monovalent vaccines. Each strain was grown in Brain Heart Infusion (Difco, Detroit, MI, USA) containing 2% Peptone, and incubated at 37°C overnight. The cultures were then centrifuged at 332g, the pellets suspended in sterile saline and their bacterial concentration determined by plating serial dilutions on blood agar. Suspensions containing  $2.5 \times 10^8$  CFU in 5 ml were inactivated with 1:5000 formaldehyde during 24 h at 37°C, and Aluminum hydroxide gel to a final concentration of 2.0mg of Al mL<sup>-1</sup> of vaccine was added. Tests for safety, sterility and purity were performed following CFR 9, § 113.100 (CFR 9, 1996).

### Mice

Isogenic Balb-c two months-old mice were randomly divided in 13 groups with four mice each. The animals of each group were inoculated subcutaneously with 1/20<sup>th</sup> of a horse dose (CFR 9, 1996) of the respective vaccine, on days 0 and 14 of the experiment. Two groups were vaccinated with commercial vaccines, following the same protocol. Blood samples were collected from each animal at 0, 14, 28, 56 and 70 days after the application of the first dose of vaccine (dpi). One group remained as unvaccinated control.

### Antibody titration

Antibodies were titrated by ELISA. Briefly, polystyrene ELISA plates (Greiner Labortechnik, Germany) were sensitized at 4°C overnight with 50µL

of inactivated bacterial suspensions containing  $0.5 \times 10^8$  cells  $\text{ml}^{-1}$  or with the commercial vaccines, suspended in carbonate-bicarbonate buffer pH 9.6. For the estimation of CRIs, pools of sera of each group of mice collected 56dpi were added and tested with homologous and heterologous antigens. To evaluate the immunogenicity of the vaccines, individual serum samples collected 0, 14, 28, 56 and 70dpi were tested with the homologous antigen. Peroxidase conjugated rabbit anti mouse immunoglobulins (Dako Co., Carpinteria, CA, USA) and ortho-phenyl dyamine were used as reagents. Optical densities were read at 450 nm in a MR 700 ELISA reader (Dynatech Labs. Inc., Chantilly, VA, USA). Optical densities of each antiserum were transformed to seroconversions dividing  $\text{OD}_{450}$  values by that of the same animal at day 0 (GIL-TURNES et al., 1999), and the means of each group calculated.

#### Cross reactivity indices

The Bilateral Cross Reactivity Indices (CRIs) among ten strains recovered from field cases and the two commercial vaccines were estimated using sera collected 56dpi by the equation  $\text{CRI} = 100 \sqrt{r \times r'}$ , where  $r$  is the quotient of the  $\text{OD}_{450}$  of serum A with antigen B and that of serum A with antigen A, and  $r'$  the quotient of the  $\text{OD}_{450}$  of serum B with antigen A and that of serum B with antigen B (ARROWSMITH, 1977). Isolates whose CRIs were  $\geq 70$  were considered of the same serogroup.

#### Western Blots.

Western blots were done using recombinant *S. equi* protein M (rSEM) produced in our laboratory and sera from mice vaccinated with experimental and commercial bacterins. Briefly, rSEM was submitted to SDS-PAGE and transferred to a 0,45m $\mu$  membrane

(Hybond-C Extra, Amersham Biosciences UK Ltd., Buckinghamshire, UK). The membranes were blocked for one hour with 5% non fat dry milk, and then 1:50 dilutions of sera from each vaccinated group were added and incubated for another hour. Peroxidase conjugated rabbit anti mouse immunoglobulins diluted 1:1000 (Dako Co., Carpinteria, CA, USA) was added and incubated for one hour. Anti-6 $\times$ His alkaline phosphatase conjugated MAb (Sigma-Aldrich) was used as positive control. Following, DAB-chromogen substrate (9mL Tris-HCl 50mM, 1mL nickel sulphate 0.3%, 10 $\mu$ L of 30% hydrogen peroxide and 6mg 3,3-diaminobenzidine tetrahydrochloride) was placed over the membranes and the reactions recorded.

## RESULTS AND DISCUSSION

*S. equi* subsp. *equi* was isolated from only thirteen (37.1%) out of thirty-five animals with typical signs of Strangles, stressing the difficulty to reach an etiological diagnosis of the disease. Atypical strains (GRANT et al., 1993), that represented 38.5% of the isolates, were recovered from three foals and two young horses belonging to three herds distanced more than 180km from one another. They fermented at least one carbohydrate, and were classified by the API 20 STREP system. Four of the five atypical strains fermented carbohydrates only in media containing serum, in agreement with previous results of TIMONEY & MUKHATAR (1993).

With the exception of strains 2 and 25, that had CRIs lower than 70 with strains 7 and 14, and 8, 26, 31 and 61, respectively, all of them from different herds, the others were antigenically homogeneous. CRIs of seven of the ten isolates tested were higher than 70 among them (Table 1), the threshold to consider strains

Table 1 - Cross reactivity indices of ten field strains of *Streptococcus equi* subsp. *equi* and two commercial vaccines.

Strains	2	6	25*	30*	31	26*	7	8*	35	14	VC A	VC B
2	100	93	75	111	80	83	67	77	76	61	44	27
6		100	83	88	103	75	83	75	71	113	41	32
25*			100	76	69	67	74	66	61	98	40	31
30*				100	96	104	120	105	94	134	43	33
31					100	88	123	90	99	92	39	23
26*						100	116	72	224	87	34	35
7							100	117	115	131	46	40
8*								100	100	103	30	29
35									100	97	41	27
14										100	33	43
VC A											100	83
VC B												100

\*, atypical strains; VC A, commercial vaccine A; VC B, commercial vaccine B.

as belonging to the same serogroup (ARROWSMITH, 1977). CRIs of 61, the lowest among field strains, were estimated for strains 2 and 14, and strains 25 and 35 (Table 1). Strains 2 and 7, and 25 with 8 and 26, had CRIs of 66 and 67, respectively. The CRI of the two commercial vaccines was 83, also showing antigenic identity, but CRIs among them and the field strains varied between 23 and 46, showing very low antigenic relationships, suggesting that the vaccines would not be able to induce acceptable immunity against field strains (Table 1).

All the vaccines were immunogenic, although those prepared with strains 14 and 35, both typical strains, induced very low seroconversions. The highest seroconversion, 9.4, was induced by strain 26, an atypical strain, 70dpi (Figure 1). The commercial vaccines and four experimental vaccines did not induce seroconversions until the animals were boosted 14dpi. The vaccines showed maximal seroconversions 70 dpi, with the exception of vaccines 14 and 35, and three experimental vaccines (2, 25 and 29), that showed maximal seroconversions 56dpi (Figure 1). Western blots (Figure 2) showed that all the experimental and one commercial vaccine induced the production of antibodies against the recombinant *S. equi* M protein (rSeM).

Although the protection induced by inactivated vaccines, using subunits or whole cells as antigens, is generally poor and does not resist to a challenge with pathogenic strains (JACOBS et al., 2000), bacterins are produced in Brazil and other

countries using strains recovered from spontaneous cases of Strangles. In our experiment we showed that some vaccines were more immunogenic than others, although they were prepared with recently isolated field strains, following the same technology and with similar antigenic concentration to the commercial vaccines. Strain 26, recovered from a foal with guttural pouch empyema, induced seroconversions that were at least twice those of the strain that followed, and more than ten times higher than the least immunogenic strain (Figure 1). On the other hand, two experimental vaccines were poorly immunogenic. The commercial vaccines were also poorly immunogenic, inducing lower seroconversions than five and four experimental vaccines, respectively. Although potency tests of commercial vaccines for Strangles are not routinely done in Brazil, the mice immunogenicity test used in our experiment could be useful to select candidate strains for vaccine production and in the quality control of bacterins.

Our results suggested that the rates of protection found in the herds studied could be related with the low immunogenicity of the commercial vaccines and with the low antigenic relationships among vaccinal and field strains.

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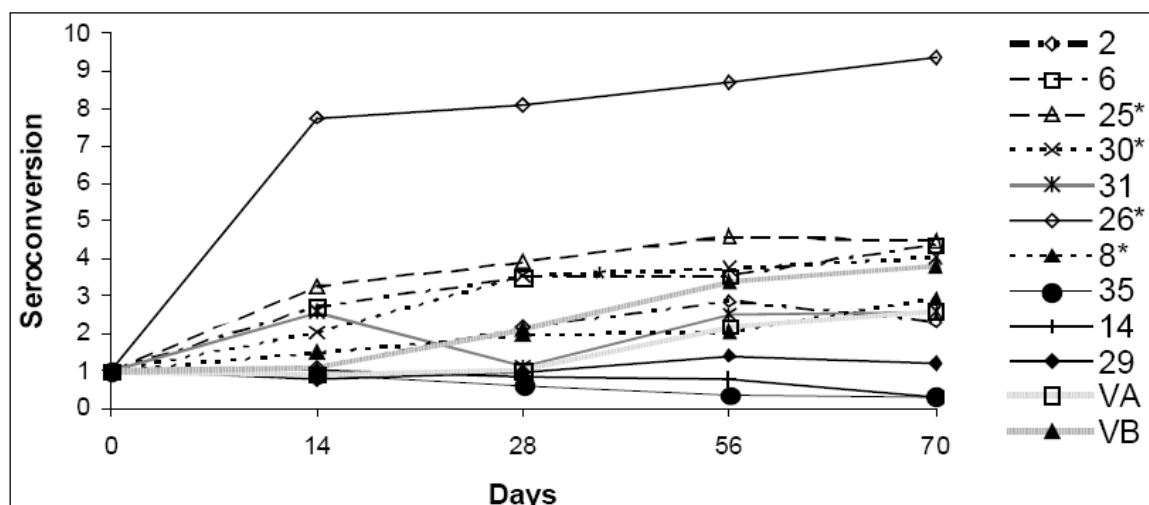


Figure 1 - Seroconversions induced in mice by vaccines prepared with field and vaccinal strains (VA, VB) of *Streptococcus equi* subsp. *equi*. (\*, atypical strains)



Figure 2 - Western blots using rSeM as antigen. 1: positive control (anti-6×His alkaline phosphatase conjugated MAb); 2: negative control; 3: strain 2; 4: strain 6; 5: strain 25\*; 6: strain 30\*; 7: strain 31; 8: strain 26\*; 9: strain 8\*; 10: strain 35; 11: strain 14; 12: strain 29; 13: Vaccine A; 15: Vaccine B. (\*, atypical strains).

## BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The animals used in this work were managed according to the requirements of the national Experimental Animals Protection Law, and with bioethics and biossecurity committee approval .

## REFERENCES

- ARROWSMITH, A.E.M. A survey of foot and mouth disease virus type O strains from Far East. **Development in Biological Standardization** , v.35, p.221-230, 1977.
- BROOKSBY, J. B. Variants in immunity: definitions for serological investigations. **Simposia series in immunological standardization**, v.8, p.1-10, 1968.
- CFR 9, Code of Federal Regulations 9. **Animals and animal products**. Standards requirements, part 1, section 113, 1996. Washington: United States Government Printing Office,1996. 1550p.
- CHANTER, N. Streptococci and enterococci as animal pathogens. **Journal of Applied Microbiology**, v.83 (suppl), p.1005-1095, 1997
- CONCEIÇÃO, FR., et al. Antigenic relationships of *Moraxella bovis* isolates recovered from outbreaks of Infectious Bovine Keratoconjunctivitis in Argentine, Brazil and Uruguay between 1983 and 2000. **Canadian Journal of Veterinary Research**. v.67, p.315-318, 2003
- EUZÉBY, J.P. **Dictionnaire de bactériologie vétérinaire**. Capturado em 28 jan. 2007. On line. Disponível na internet: <http://www.bacterio.cict.fr/bacdico/ss/equi.html>
- FARROW, J.A.E., COLLINS, M.D. Taxonomic studies on streptococci serological group C, G and L and possible related taxa. **Systematic and Applied Microbiology** , v.5, p.483-493, 1984.
- FLOCK, M., et al. Recombinant *Streptococcus equi* proteins protect mice in challenge experiments and induce immune response in horses. **Infection and Immunity**. v.72, n.6, p. 3228-3236, 2004. Disponível em: <http://iai.asm.org/cgi/content/abstract/72/6/3228>. Doi: 10.1128/IAI.72.6.3228-3236.2004.
- GRANT, S.T. et al. Laboratory diagnosis of strangles and the isolation of atypical *Streptococcus equi*. **Veterinary Record**, v.133, p.215-216, 1993.
- GIL TURNES, C., et al. DNA inoculation with a plasmid vector carrying the *faeG* adhesin gene of *Escherichia coli* K88ab induced immune response in mice and pigs. **Vaccine**. v.17, p.2089-2095, 1999. Disponível em: [http://dx.doi.org/10.1016/S0264-410X\(98\)00384-3](http://dx.doi.org/10.1016/S0264-410X(98)00384-3). Doi : 10.1016/S0264-410X(98)00384-3.
- HARRINGTON, D.J. et al. The molecular basis of *Streptococcus equi* infection and disease. **Microbes and Infection**. n.4, p. 501-510, 2002. Disponível em: [http://www.sciencedirect.com/science?\\_ob=ArticleURL&\\_udi=B6VFN-45CW9F8-D&\\_user=687358&\\_rdoc=1&\\_fmt=&\\_orig=search&\\_sort=d&view=c&\\_acct=C000037899&\\_version=1&\\_urlVersion=0&\\_userid=687358&md5=78c2fac5ab8d0936186abf9951d1637c](http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6VFN-45CW9F8-D&_user=687358&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_acct=C000037899&_version=1&_urlVersion=0&_userid=687358&md5=78c2fac5ab8d0936186abf9951d1637c). Doi: 10.1016/S1286-4579(02)01565-4.
- JACOBS, A.A., et al. Investigations towards an efficacious and safe strangles vaccine: submucosal vaccination with a live attenuated *Streptococcus equi*. **Veterinary Record**, v.20, n.147, p.563-567, 2000.
- KUWAMOTO, Y. et al. Microplate sugar-fermentation assay distinguishes *Streptococcus equi* from other streptococci of

Lancefield's group C. **Equine Veterinary Science**, v.12, n. 2, p.47-49, 2001.

OLIVEIRA, A.O.; GIL-TURNES, C. Studies on the antigenic relationships of six adherent isolates of *Bordetella bronchiseptica*. **Veterinary Microbiology**, v.18, p.327-333, 1988.

PEREIRA, H. Subtyping of Foot and Mouth Disease Virus. **Development in Biological Standardization**, v.35, p.167-174, 1977.

SWEENEY, C.R. *Streptococcus equi*. In: SMITH, B.P. **Tratado de medicina interna de grandes animais**. São Paulo: Manole, 1993. p.531-533.

TIMONEY, J.F.; MUKHATAR, M.M. The protective M proteins of the equine group C streptococci. **Veterinary Microbiology**, n.37, p.389-395, 1993.

TIMONEY, J.F. et al. Comparison of the sequences and functions of *Streptococcus equi* M-like proteins SeM and SzPSe. **Infection and Immunity**, n.65, p.3600-3605, 1997.